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Interleukin-10 limits increased blood pressure and vascular RhoA/Rho-kinase signaling in angiotensin II-infused mice

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ABSTRACT

Aims: Interleukin-10 (IL-10) is a multi-functional cytokine with potent anti-inflammatory properties. We hypothesized that IL-10 limits increased RhoA/Rho-kinase signaling and vascular reactivity in arteries from angiotensin II (Ang II) hypertensive mice.
Main methods: Wild type (WT) and IL-10 knockout ($^{-/-}$) mice were infused with Ang II (90 ng/min) for 14 days. Additionally, WT mice were infused with Ang II and simultaneously infused with exogenous IL-10 (0.5 ηg/min, 14 days). Aortic rings were mounted in a myograph and concentration-response curve to phenylephrine (PE) were evaluated.
Key findings: After Ang II infusion, blood pressure responses, but not maximal contraction to PE, was greater in IL-10$^{-/-}$ mice, compared to WT. Rho-kinase inhibition (Y-27632; 10 μM) resulted in a more evident reduction of PE-induced contraction in WT hypertensive mice, when compared to IL-10$^{-/-}$ hypertensive mice. IL-10 exogenous infusion prevented the blood pressure increase in Ang II-infused WT mice. The augmented PE-contraction observed in aorta from WT mice infused with Ang II was also prevented by exogenous infusion of IL-10. Additionally, Rho-kinase inhibition (Y-27632; 10 μM) abolished the differences in the contractile response to PE between these groups.
Significance: These results demonstrate that IL-10 counteracts both the pressoric activity of Ang II as well as vascular dysfunction associated with hypertension, partially, modulating the RhoA-Rho kinase pathway. Strategies to enhance IL-10 levels during hypertension may enhance the benefits provided by regular treatments.

1. Introduction

Low-grade inflammation is a player on the pathophysiology of cardiovascular diseases. In this regard, it has been established that increased production of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), modulates vascular function by increasing contractile pathways and also, by decreasing endothelium-dependent vascular relaxation through distinct mechanisms [1].

Interleukin-10 (IL-10) is a multi-functional cytokine with potent anti-inflammatory properties. The main function of IL-10 is to limit and terminate inflammatory responses, by inhibiting a broad array of immune parameters [2,3]. During the course of inflammation, IL-10 is produced by type 2 helper (Th2) T cells, B cells, monocytes and macrophages [2].

Stimulation of vascular smooth muscle cell (VSMC) with angiotensin II (Ang II) promotes pro-inflammatory injury in the vasculature, by mechanisms related with the increased production of pro-inflammatory cytokines, whereas the release of anti-inflammatory cytokines, such as IL-10, is reduced [4–7]. Interestingly, it was found that when hypertensive patients were treated with drugs that interfere with Ang II actions, such as Ang II receptor 1 (AT1) receptor antagonists or Ang II-converting enzyme (ACE) inhibitors, serum IL-10 levels were augmented, compared to untreated hypertensive patients [3,8,9]. These findings suggest the interplay between Ang II and IL-10 during hypertension.

It was shown that IL-10 improves endothelium-dependent relaxation by inhibiting NADPH oxidase activity in Ang II-hypertensive rats, improving their blood pressure [10]. However, the direct interplay between IL-10 and VSMC contractility during hypertension remains an unexplored area.

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Increased activation of Ang II-related pathways in VSMC culminates in increased vascular contractility, a hallmark of hypertension [11,12]. Increased contraction in VSMC, induced by Ang II, has been associated with enhanced activation of the RhoA/Rho-kinase signaling pathway, a system which regulates calcium-sensitization. In fact, a role for enhanced RhoA/Rho kinase activity has been reported both in experimental and clinical hypertension [11,13].

As previously mentioned, Ang II promotes pro-inflammatory injury in the vasculature and the antagonism of its action lowers blood pressure, improves vascular function and elevates serum levels of IL-10 [9]. Considering these evidences, we hypothesized that IL-10 therapy limits increased RhoA/Rho-kinase signaling in arteries from Ang II-hypertensive mice, improving both increased vascular contractility and augmented blood pressure during hypertension.

The overall strategy was to evaluate the endogenous and exogenous role of IL-10 actions in the blood pressure regulation and vascular contractile function in Ang II-hypertensive mice.

2. Methods

2.1. Animals

Male B6.129P2-Il10^{tm1Cgn}/J (IL-10$^{-/-}$) mice and their control C57BL/6 J mice [wild type (WT), The Jackson Laboratory, Maine, US], with 10–12 weeks, were used in this study. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Georgia Health sciences university Committee on the Use of Animals in Research and Education. The animals were housed four per cage on 12-h light/dark cycle and fed a standard chow diet with water ad libitum. Animals were anesthetized with isoflurane via a nose cone for surgical procedures (initially with 5% and then maintained at 2.0% in 100% oxygen). Osmotic mini pumps (0.25 μl per hour – 14 days – model 1002, Alzet Co., Cupertino, CA) were implanted subcutaneously.

The first set of experiments was designed to evaluate the role of endogenous IL-10 in hypertensive mice. Therefore, IL-10$^{-/-}$ and WT mice were infused with vehicle only (saline) or with Ang II (90 ηg·min^{-1}) for a period of 14 days.

The second set of experiments was designed to evaluate the role of exogenous IL-10 in hypertensive mice. C57BL/6 J mice were infused with vehicle (saline) or Ang II (90 ng.min^{-1}), and simultaneously infused (additional mini-pump), with or without recombinant mouse IL-10 (0.5 ηg.min^{-1}), for a period of 14 days.

In these two sets of experiments, mean arterial pressure was assessed by catheterization, as described following.

2.2. Blood pressure recording

At the end of experiment mice were anesthetized with isoflurane via a nose cone for surgical procedures (initially with 5% and then maintained at 2.0% in 100% oxygen). Following anesthesia, a sterile catheter PE-10 tube was inserted into the carotid artery. The incision was closed with a sterile 6–0 Ethicon Ophthalmic suture. The catheter was secured on the back of the mouse to avoid any biting of the tube by the mouse. All surgeries were conducted under aseptic and sterile conditions to avoid any chances of infection. Mice received one unique dose of tromethamine (1 g·kg^{-1} i.m.) after the surgery. Once the mouse had completely recovered from anesthesia and from the stress manipulation (2 h after surgery), the catheter was connected to a transducer to record mean arterial pressure. Recording measurements of mean arterial pressure were made for 3 to 4 h. Subsequently, blood was collected and the mice were killed in a CO_2 chamber, and the aorta was isolated for functional and molecular studies.

2.3. Vascular functional studies

Thoracic aortas were rapidly excised and placed in ice-cold physiological salt solution (PSS), and carefully dissected. Endothelium was mechanically removed by rubbing the artery with a metallic pin. Aortas were mounted as ring preparations (4 mm in length) in standard organ chambers for isometric tension recording by a PowerLab 8/SP data acquisition system (ADInstruments Pty Ltd., Colorado Springs, CO). The segments were adjusted to maintain a passive force of 5 mN. Aortas were equilibrated for 60 min in PSS at 37 °C, and continuously bubbled with a mix of 5% CO_2 and 95% O_2. Arterial integrity was assessed by stimulation of vessels with high potassium solution (KCl, 120 mM). After washing and a new stabilization, the absence of endothelium was assessed by contracting the segments with phenylephrine (PE; 1 μM) followed by acetylcholine (ACh; 10 μM). The absence of a relaxation-response to ACh stimulation was taken as evidence of endothelium removal. Concentration-response curve to PE (1 nM to 100 μM) were performed in presence or absence of Y27632 (10 μM, 40 min), a Rho kinase inhibitor.

2.4. Western blot Analysis

Proteins (40 μg) extracted from aorta were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween (TBS-T) for 1 h at 24 °C. Membranes were then incubated with primary antibodies overnight at 4 °C. Antibodies were as follows: RhoA (Abcam, 1:1000), ROCK-α and ROCK-β (BD Transduction, 1:500). On next day membranes were removed from primary antibodies and washed with TBS-T. Membranes were treated with secondary antibody for one hour. After incubation with respective secondary antibodies, signals were visualized using chemiluminescence and images were captured using Alpha Imager from Alpha Innotech (San Leandro, CA). All blots were stripped and probed with anti-β-actin antibody (Sigma, 1:20,000), to ensure equal protein loading. Results were normalized to β-actin protein and expressed as arbitrary units.

2.5. IL-10 level

Plasmatic level of mouse IL-10 was determined by sandwich enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Minneapolis, MN).

2.6. Drugs and solutions

Physiological salt solution (PSS) of the following composition was used: 130 mM NaCl, 14.9 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4 \cdot 7H_2O$, 5.5 mM glucose, 1.56 mM $CaCl_2 \cdot 2H_2O$ and 0.026 mM EDTA. Ang II was purchased from Phoenix Pharmaceutical Inc. (Burlingame, CA). Recombinant mouse IL-10 was purchased by BD Bioscience (San Jose, California). PE and ACh were purchased from Sigma Chemical Co. (St. Louis, MO). Y-27632 was purchased from Tocris (Ellisville, MO). All reagents were of analytical grade. Stock solutions were prepared in ultrapure water or saline (Ang II and IL-10).

2.7. Data analysis

Results are presented as mean ± standard error of the mean (SEM). Contractions were recorded as changes in the displacement (mN) from baseline, normalized by KCl contraction and are represented as percentage of KCl-induced contraction, for *n* experiments. Concentration-response curves were fitted using a nonlinear interactive fitting program. Curve-fit parameters were used to fit a sigmoid curve and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or Emax) and -log EC50 (or pD2). Statistical

analysis was performed using two-way analysis of variance plus Bonferroni post-hoc analysis to compare the concentration-responses curves between all the groups. Statistically significant differences were calculated by ANOVA. Values of $p < 0.05$ were considered a statistically significant difference.

3. Results

3.1. Effect of IL-10 and Ang II on blood pressure

After 14 days, Ang II infusion augmented mean arterial pressure [MAP (mmHg)] both in WT and IL-$10^{-/-}$ mice. However, changes in MAP after Ang II infusion was greater in IL-$10^{-/-}$ mice, compared to WT. No differences were observed in MAP between in vehicle-infused WT or vehicle-infused IL-$10^{-/-}$ mice.

Exogenous IL-10 infusion did not result in significant MAP changes in WT mice, after 14 days. However, mice infused simultaneously with exogenous IL-10 and Ang II showed reduced MAP, compared to WT mice infused with Ang II or IL-$10^{-/-}$ mice infused with Ang II (Fig. 1A).

After 14 days, plasma IL-10 levels ($pg \cdot mL^{-1}$) were smaller in Ang II-infused WT mice, compared to vehicle-infused WT mice (4.9 ± 2.3 vs. 10.6 ± 1.5, respectively). Exogenous IL-10 infusion increased plasma IL-10 levels both in vehicle-infused WT (18.3 ± 2.8) and in Ang II-infused WT mice [24.4 ± 2.3; (Fig. 1B)].

3.2. Effect of IL-10 on PE-induced vasoconstriction

Aortas from Ang II-infused WT mice displayed augmented PE-induced contraction, compared to vehicle-infused WT mice [E_{max} 212 ± 9 vs. 175 ± 9%, respectively; (Fig. 2A)].

Ang II-infused IL-$10^{-/-}$ mice displayed augmented PE-induced contraction (E_{max} 225 ± 6%) when compared to vehicle-infused IL-$10^{-/-}$ mice [E_{max} 189 ± 8%; (Fig. 2B)]. No differences were observed

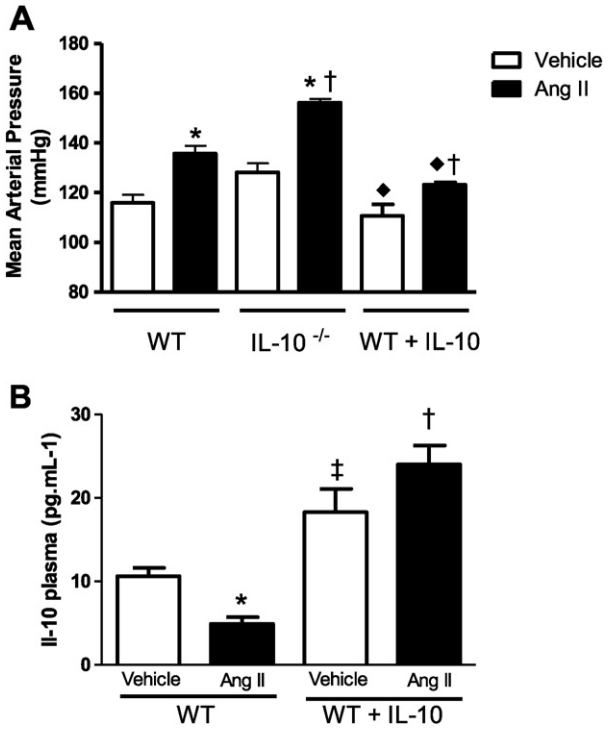

Fig. 1. IL-10 prevents Ang II pressure response. A) Mean arterial pressure changes upon vehicle or Ang II infusion observed in WT mice, IL-$10^{-/-}$ mice or WT mice infused with exogenous IL-10 after 14 days (n = 5–9). B) IL-10 plasma levels in WT mice infused or not with Ang II for 14 days, with or without simultaneous infusion of exogenous IL-10 (n = 6). * $P < 0.05$ vs. respective vehicle; ‡ $P < 0.05$ vs. WT + vehicle; † $P < 0.05$ vs. WT + Ang II and ♦ vs. respective IL-$10^{-/-}$ group.

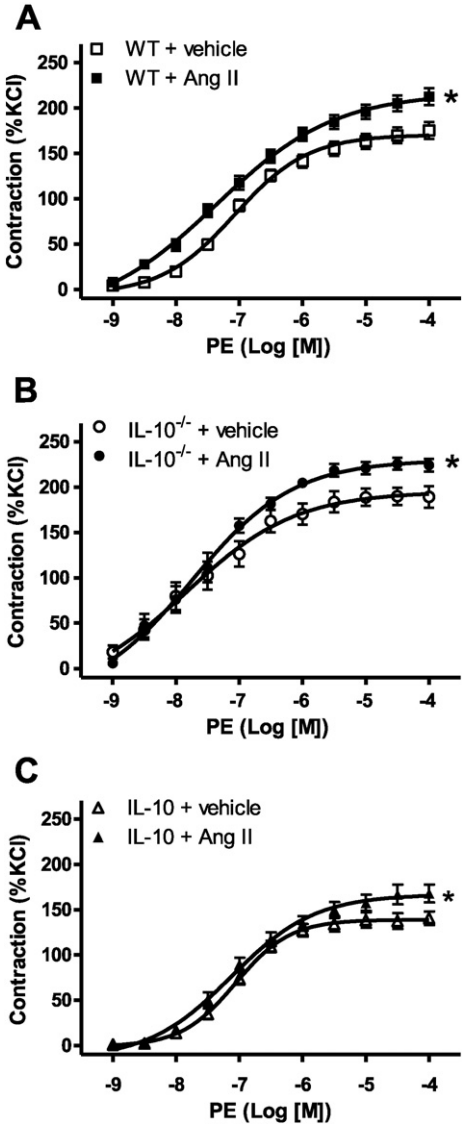

Fig. 2. Effect of IL-10 on PE-induced contractile response in aorta form Ang II-infused mice. Contractile response in aorta from mice infused (closed symbols) or not (open symbols) with Ang II: A) WT mice; B) IL-$10^{-/-}$ mice; C) WT mice infused with exogenous IL-10; (n = 6–10)* $P < 0.05$ vs. respective vehicle.

in PE-contractile response between aortas from WT and IL-$10^{-/-}$ mice infused with vehicle suggesting that, physiologically, contractile properties are not affected in these mice. Ang II-infused IL-$10^{-/-}$ mice showed augmented sensitivity to PE compared to Ang II-infused WT mice (Table 1). No differences were observed in KCl-induced contraction between groups.

Augmented PE-induced contraction persisted in aortas from WT co-infused with exogenous IL-10 and Ang II (E_{max} 167 ± 5%), when compared to aortas from WT infused exclusively with exogenous IL-10 [(E_{max} 139 ± 3%), Fig. 2B]. However, exogenous IL-10 infusion decreased PE-induced contraction, when compared to aortas from WT mice infused with vehicle (Table 1). Additionally, when exogenous IL-10 was simultaneously infused with Ang II, a smaller contractile response to PE was observed, when compared to aortas from WT mice infused with Ang II alone (Table 1).

In order to investigate if IL-10 decreases PE-contraction by modulating the RhoA/Rho kinase pathway, incubations with a Rho kinase inhibitor (Y-32627, 10 μM) were performed.

Under Rho-kinase inhibition condition, maximum contraction to PE were reduced both in Ang II and vehicle-infused WT mice (E_{max} 115 ±

Table 1
Emax (%KCl) and pD_2 values for phenylephrine induced contraction in the presence or absence of Y-27632.

				Y-27632	
	KCl (mN)	Emax	pD_2	Emax	pD_2
WT + vehicle	7,1 ± 0.4	175 ± 9	7.1 ± 0.1	121 ± 14	6.7 ± 0.3
WT + Ang II	7,0 ± 0.5	212 ± 9*	7.4 ± 0.2*	115 ± 20	6.8 ± 0.6
IL-$10^{-/-}$ + vehicle	7,3 ± 0.8	189 ± 8	7.8 ± 0.4‡	114 ± 18	7.2 ± 0.3‡
IL-$10^{-/-}$ + Ang II	7,2 ± 0.8	225 ± 6*	7.7 ± 0.2†	156 ± 06*†	6.7 ± 0.4 *
WT + IL-10	7,0 ± 0.3	139 ± 3‡	7.2 ± 0.1	57 ± 10‡	6.6 ± 0.2
WT + IL-10 + Ang II	7,3 ± 0.6	167 ± 5* †	7.1 ± 0.1†	73 ± 06†	6.7 ± 0.2

Concentration-response curves to phenylephrine (PE) and single stimulation with high potassium solution (KCl, 120 mM) were performed in aorta from mice, in the absence or in the presence of Y-27632 (10 μmol/L, for 40 min). Data are mean ± SEM (n = 6). * P < 0.05 vs. respective vehicle group; † P < 0.05 vs. WT + Ang II; ‡ P < 0.05 vs. WT + vehicle.

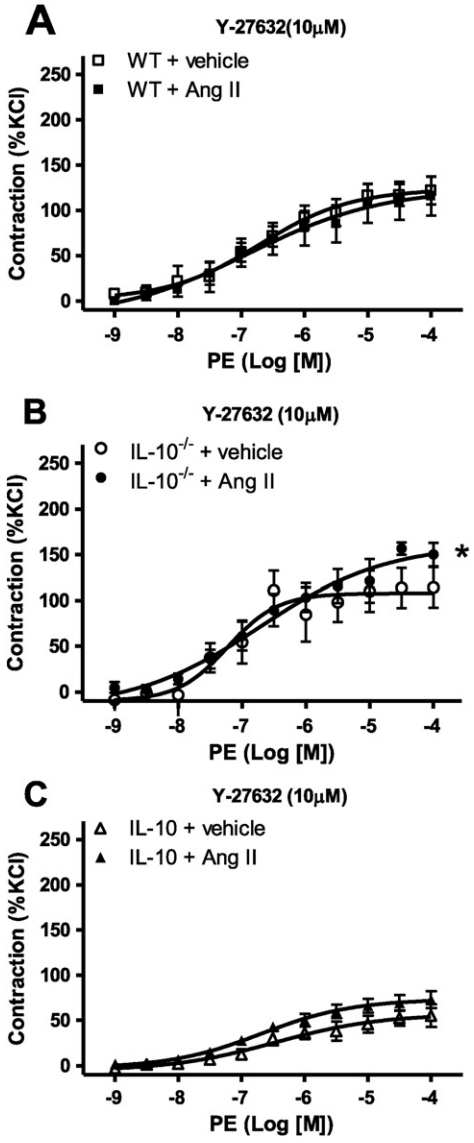

Fig. 3. IL-10 decreased contraction in aorta form Ang II-infused mice through rho kinase-dependent mechanism. Contractile response in aorta from mice infused (closed symbols) or not (open symbols) with Ang II, in the presence of the rho kinase inhibitor, Y-27632 (10 μM): A) WT mice; B) IL-$10^{-/-}$ mice; C) WT mice infused with exogenous IL-10; (n = 5–6). * P < 0.05 vs. respective vehicle.

20 vs. 121 ± 14%, respectively) and differences previously observed between these groups were abolished after Rho-kinase inhibition (Fig. 3A).

Aortas from Ang II- or vehicle-infused IL-$10^{-/-}$ mice incubated with Rho-kinase inhibitor resulted in smaller PE-induced contraction in both groups (E_{max} 156 ± 6 vs. 114 ± 18%, respectively). However, Rho-kinase inhibition was not able to completely abolish differences in the contractile response to PE observed in aortas from Ang II-infused IL-$10^{-/-}$ mice, compared to vehicle-infused IL-$10^{-/-}$ mice (Fig. 3B).

In addition, after Rho-kinase inhibition, PE-induced contractions were further reduced in aortas from WT mice infused simultaneously with Ang II and exogenous IL-10 or only infused with IL-10 (73 ± 6 vs. 57 ± 10%, respectively). In this case, differences between these groups were abolished after Rho-kinase inhibition (Fig. 3C).

3.3. Effect of IL-10 on RhoA and Rho kinases protein expression

RhoA expression was increased after Ang II infusion, both in aortas from WT or IL-$10^{-/-}$ mice, when compared to their respective vehicle-infused control. WT mice infused with Ang II did not display changes on ROCK-α expression. However, ROCK-α expression was augmented in aortas from IL-$10^{-/-}$ mice infused with Ang II or vehicle. ROCK-β was augmented in aortas from Ang II-infused WT mice. The expression of ROCK-β was further increased in aortas from IL-$10^{-/-}$ mice, especially after Ang II infusion (Fig. 4A).

Expression of RhoA was greater in aortas from Ang II-infused WT mice, compared to vehicle-infused WT mice. Simultaneous infusion of Ang II and IL-10 in WT mice prevented RhoA increased expression. Furthermore, infusion of IL-10 alone did not change RhoA expression. IL-10 exogenous infusion or Ang II infusion did not affect expression of ROCK-α. However, IL-10 simultaneously infused with Ang II in WT mice prevented the augmented expression of ROCK-β, which was observed in aortas from Ang II-infused WT mice (Fig. 4B).

4. Discussion

The major findings of this study were: (1) endogenous IL-10 contributes to blood pressure regulation under Ang II infusion; (2) exogenous IL-10 infusion was effective to increase IL-10 circulating levels and was able to prevent Ang II-pressoric actions, but did not affect blood pressure control in physiological conditions; (3) under Ang II infusion, the absence of IL-10 resulted in increased contractile response to PE, and in WT mice, exogenous infusion of IL-10 prevented increased contraction in aortas from Ang II-infused mice; (4) increased response to PE in aortas from Ang II-infused mice were decreased by Rho-kinase inhibition and (6) IL-10 seems to negatively modulate RhoA/Rho kinase expression.

Initially, we aimed to further investigate if IL-10 regulates blood pressure. To test this, we used the Ang II-hypertension model. Ang II regulates blood pressure by several mechanisms including vasoconstriction, release of reactive oxygen species, production of pro-inflammatory mediators, among others [12]. Using direct blood pressure measurement, we observed that IL-10 knockout mice displayed similar MAP at basal condition, compared to their respective control mice. These results are in agreement with recent studies, where systolic blood pressure was measured in conscious mice using tail-cuff [10,14].

Upon Ang II infusion, changes in mean arterial pressure were greater in IL-10 knockout mice, compared to control mice, showing that endogenous IL-10 limits Ang II-pressoric effect. In addition, exogenous IL-10 infusion was able to prevent increases in blood pressure after Ang II infusion. Kassan and colleagues observed similar effects in diastolic blood pressure, where Ang II-hypertensive rats were simultaneously infused with exogenous IL-10 (1000 ng/mouse, for 14 days). They attribute that IL-10 prevents the pressor effects of Ang II, mainly by inhibiting activation of NADPH oxidase and by improving nitric oxide bioavailability, and therefore, by improving endothelial function [10].

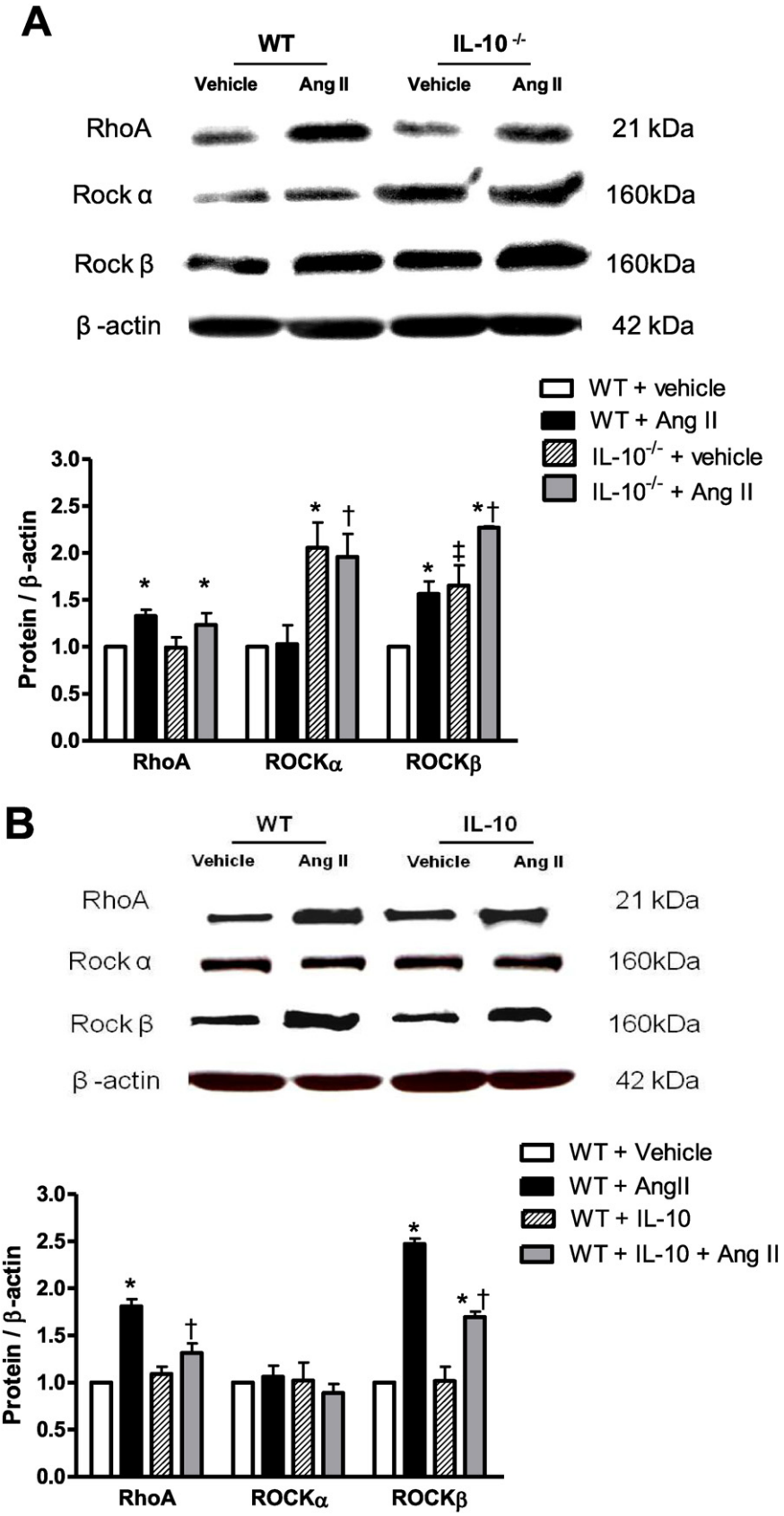

Fig. 4. Vascular expression of RhoA and rho kinases α and β is modulated by IL-10. RhoA, ROCK-α and ROCK-β expression in aortas from mice infused or not with Ang II: A) WT mice and IL-$10^{-/-}$ mice; B) WT mice infused with or without exogenous IL-10; (n = 5–6). * $P < 0.05$ vs. respective vehicle; ‡ $P < 0.05$ vs. WT + vehicle; † $P < 0.05$ vs. WT + Ang II.

Considering that other mechanisms play a role in blood pressure control, we decided to evaluate other pathways modulated by IL-10, with potential protective activity both in vascular function and in blood pressure regulation. Our results demonstrated increased vascular contractile response to PE, in aortas from hypertensive mice. The mechanisms responsible to elevate vascular contractile responsiveness in hypertension are complex. However, it is clear that the balance in cellular signaling mechanisms that promote contraction and relaxation of vascular smooth muscle cells is disrupted to favor vasoconstriction and the increased peripheral resistance is caused, in part, by an alteration in vascular smooth muscle that makes it more sensitive to normal stimuli [11], contributing to augmented blood pressure.

The protective role of IL-10 on the vascular function was initially demonstrated, addressing mostly the protective effect of this cytokine in the endothelial function. It was shown that IL-10 knockout mice, upon Ang II infusion, displays decreased endothelium-dependent relaxation, mainly due to augmented oxidative stress production [14]. Here, we show a direct effect of Il-10 on vascular smooth muscle cells. We showed that upon Ang II infusion, absence of endogenous IL-10 resulted in augmented vascular constriction; whereas exogenous IL-10 infusion prevented this effect, implying that IL-10 is able to regulate vascular smooth muscle contraction and to partially prevent Ang II actions.

Smooth muscle cells contain Ca^{2+}-independent mechanisms to regulate contractility. The process of force generation is mediated by myosin light chain kinase (MLCK) activation, and subsequent actin-myosin cross-bridging, but the process of force maintenance is mediated in a Ca^{2+}-independent manner, and the RhoA/Rho kinase pathway has been directly linked to this event [12,13]. Indeed, the increased vascular reactivity in Ang II-induced hypertension is associated with enhanced activation of the RhoA/Rho-kinase signaling pathway [15,16]. Rho-kinase antagonist Y-27632 lowers blood pressure in several rat models of experimental hypertension [13]. In addition, increased expression of Rho-kinase protein in blood vessels from hypertensive rats has been demonstrated [17–19]. Therefore, we focused our attention to investigate if IL-10 plays a modulatory role in the RhoA/Rho kinase pathway during Ang II-induced hypertension.

In our study, Rho-kinase inhibition abolished augmented contractile response in aortas from mice infused with Ang II and IL-10, compared to vehicle and also reduced the differences between IL-10 knockout mice infused with or without Ang II, suggesting that IL-10 may negatively regulates the activation of the RhoA/Rho-kinase pathway. These data is supported by our molecular experiments, where we found that in mice aorta, IL-10 negatively modulates RhoA and Rho-kinases expression, upon Ang II-stimulation.

On the top of modulating the activation of the RhoA/Rho-kinase pathway on vascular smooth muscle cells and endothelial cells [14], it is possible that IL-10 also regulates other pathways that are over activated during Ang II hypertension. IL-10 may be acting directly on inflammatory circulating cells, decreasing their activation and therefore contributing to decreased production of pro-inflammatory cytokines. In this regard, it is well known that during hypertension, inflammatory cells, including macrophages and neutrophils, infiltrate the vasculature and generate local inflammatory response, further contributing to vascular dysfunction [20].

One additional possible mechanism by which IL-10, in part, regulates Ang II-induced hypertension is that IL-10 is produced after pro-inflammatory mediators, such as IL-6 and TNF-α and therefore, IL-10 has an important function to prevent and limit an excessive response [21–23]. Cytokines appears to modulate blood pressure also in the central neuron system level. It was recently shown that IL-10 is able to reduce the excitation of hypothalamic neurons induced by Ang II, by inducing I_{Kv} currents [24,25]. The disruption of the balance between anti- and pro-inflammatory cytokines also modulates vascular function, and therefore, influencing blood pressure control [26].

In summary, we showed that endogenous and exogenous IL-10 counteracts both the pressoric activity and vascular dysfunction associated with Ang II-induced hypertension. Since the principal function of IL-10 is to limit and terminate inflammatory responses, it may also provide a novel therapeutic target for the treatment of hypertension and we propose that strategies to elevate IL-10 may work synergistically with existent therapies to improve the outcome of hypertension.

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